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(54) Titre : COMPOSITIONS PHARMACEUTIQUES A BASE DE VEROTOXINE ET TRAITEMENTS MEDICAUX QUI
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(54) Title: VEROTOXIN PHARMACEUTICAL COMPOSITIONS AND MEDICAL TREATMENTS THEREWITH

(57) **Abrégé/Abstract:**

Pharmaceutical compositions comprising known verotoxins, particularly, verotoxin 1 and their pentameric subunit B, have been found to be useful in the treatment of mammalian neoplasia, particularly, brain cancer, ovarian cancer, breast cancer and skin cancer. Although verotoxin 1 has previously been shown to have anti-neoplastic activity in vivo, non-lethal doses of verotoxin 1 have been shown to be therapeutically anti-neoplastic in vivo. Use of a sensitizer, such as sodium butyrate, enhances the efficacy of verotoxins and their subunit B.

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Abstract of the Disclosure

5 Pharmaceutical compositions comprising known verotoxins, particularly, verotoxin 1 and their pentameric subunit B, have been found to be useful in the treatment of mammalian neoplasia, particularly, brain cancer, ovarian cancer, breast cancer and skin cancer. Although verotoxin 1 has previously been shown to have anti-neoplastic activity in vitro, non-lethal doses of verotoxin 1 have been shown to be therapeutically anti-neoplastic in vivo. Use of a sensitizer, such as sodium butyrate, enhances the efficacy of verotoxins and their subunit B.

VEROTOXIN PHARMACEUTICAL COMPOSITIONS AND
MEDICAL TREATMENTS THEREWITH

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Field of the Invention

10 This invention relates to verotoxin pharmaceutical compositions and to
methods of treating mammalian neoplasia, particularly, brain, ovarian and skin cancers,
therewith.

Background to the Invention

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 Bacteriocins are bacterial proteins produced to prevent the growth of competing
microorganisms in a particular biological niche. A preparation of bacteriocin from a particular
strain of E. coli (HSC₁₀) has long been shown to have anti-neoplastic activity against a variety
of human tumour cell lines in vitro (1,2). This preparation, previously referred to as PPB
20 (partially purified bacteriocin (2)) or ACP (anti-cancer proteins (2)) was also effective in a
murine tumour model of preventing metastases to the lung (2).

25

 Verotoxins, also known as SHIGA-like toxins, comprise a family known as
Verotoxin 1, Verotoxin 2, Verotoxin 2c and Verotoxin 2e of subunit toxins elaborated by some
strains of E. coli (3). These toxins are involved in the etiology of the hemolytic uremic
syndrome (3,4) and haemorrhagic colitis (5). Cell cytotoxicity is mediated via the binding of
the B subunit of the holotoxin to the receptor glycolipid, globotriaosylceramide, in sensitive
cells (6).

30

 The verotoxin family of E. coli elaborated toxins bind to the globo series
glycolipid globotriaosylceramide and require terminal gal α -1-4 gal residue for binding. In
addition, VT2e, the pig edema disease toxin, recognizes globotetraosylceramide (Gb₄)

containing an additional B 1-3 linked galNAc residue. These glycolipids are the functional receptors for these toxins since incorporation of the glycolipid into receptor negative cells renders the recipient cells sensitive to cytotoxicity. The toxins inhibit protein synthesis via the A subunit - an N- glycanase which removes a specific adenine base in the 28S RNA of the 60S RNA ribosomal subunit. However, the specific cytotoxicity and specific activity is a function of the B subunit. In an in vitro translation system, the verotoxin A subunit is the most potent inhibitor of protein synthesis yet described, being effective at a concentration of about 8 pM. In the rabbit model of verocytotoxemia, pathology and toxin targeting is restricted to tissues which contain the glycolipid receptor and these comprise endothelial cells of a subset of the blood vasculature. Verotoxins have been strongly implicated as the etiological agents for hemolytic uremic syndrome and haemorrhagic colitis, microangiopathies of the glomerular or gastrointestinal capillaries respectively. Human umbilical vein endothelial cells (HUVEC) are sensitive to verotoxin but this sensitivity is variable according to cell line. Human adult renal endothelial cells are exquisitely sensitive to verotoxin in vitro and express a correspondingly high level of Gb₃. However, HUS is primarily a disease of children under three and the elderly, following gastrointestinal VTEC infection. It has been shown that receptors for verotoxin are present in the glomeruli of infants under this age but are not expressed in the glomeruli of normal adults. HUVEC can be sensitized to the effect of verotoxin by pretreatment by tumour necrosis factor which results in a specific elevation of Gb₃ synthesis (7,8). Human renal endothelial cells on the other hand, although they express high levels of Gb₃ in culture, cannot be stimulated to increase Gb₃ synthesis (8). It has been suggested that the transition from renal tissue to primary endothelial cell culture in vitro results in the maximum stimulation of Gb₃ synthesis from a zero background (9). We therefore suspect that HUS in the elderly is the result of verotoxemia and a concomitant stimulation of renal endothelial cell Gb₃ synthesis by some other factor, eg. LPS stimulation of serum α TNF. Thus under these conditions, the majority of individuals (excepting the very young) would not be liable to VT induced renal pathology following systemic verotoxemia.

It has also been shown that the verotoxin targets a sub-population of human B cells in vitro (10). These Gb₃ containing B cells are found within the germinal centres of lymph nodes (11). It has been proposed that Gb₃ may be involved in a germinal centre homing by

CD19 positive B cells (12) and that Gb₃ may be involved in the mechanisms of antigen presentation (13).

Elevated levels of Gb₃ have been associated with several other human tumours (14-16), but ovarian tumours have not been previously investigated. Gb₃ is the p^k blood group antigen (17). Tissue surveys using anti-p^k antisera have shown that human ovaries do not express this glycolipid (18, 19). Sensitivity to VT1 cytotoxicity in vitro has been shown to be a function of cell growth, the stationary phase cells being refractile to cytotoxicity (20). The sequence homology between the receptor binding B subunit and the human $\alpha 2$ -interferon receptor and the B cell marker CD19 suggests that expression of Gb₃ is involved in the mechanism of $\alpha 2$ -interferon and CD19 signal transduction (12). On surface ligation, Gb₃ has been shown to undergo a retrograde intracellular transport via the rough endoplasmic reticulum to the nuclear membrane (21).

The astrocytoma is the most common primary human brain tumour. The majority of astrocytomas are malignant neoplasms which infiltrate diffusely into regions of normal brain. Despite the advent of promising adjuvant therapies and drugs which have impacted positively on patient survival in other tumor types in recent times, no such promising therapy has yet been found for the patient with a malignant astrocytoma. The median survival for patients with glioblastoma multiforme, the most malignant form of astrocytoma, is approximately 12 months and accordingly, it is imperative that new therapeutic treatments for malignant astrocytomas be found.

VTs consist of a 30kDa enzymatic A subunit which is capable of inhibiting protein synthesis. The A subunit is noncovalently associated with a pentameric 7kDa B subunit array which binds to Gb₃.

In addition to the cytotoxic effects of VTs on a wide range of cells by the A subunit inhibition of protein synthesis, recent evidence suggests that VT1, and the receptor binding B subunit alone, also induce morphological changes and DNA fragmentation characteristic of apoptosis in Gb₃-positive cells (22, 23).

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Although anti-neoplastic effects of bacterial preparations have been known for over twenty years, the neoplastic effect of verotoxin per se has, to-date, remained unknown. As

25 a result of extensive investigations, we have discovered that verotoxin, particularly Verotoxin 1, is an active component within the ACP and that purified Verotoxin 1 has potent anti-neoplasia effect in vitro and in vivo. Most surprisingly, we have found effective in vivo anti-cancer treatments of human beings commensurate with non-toxic administered dosages.

Summary of the Invention

5 It is an object of the present invention to provide a pharmaceutical composition for the treatment of mammalian neoplasia and, particularly, brain, skin cancer and ovarian cancer.

It is a further object of the present invention to provide a method of treating mammalian neoplasia, particularly, skin, brain and ovarian cancers.

10 Accordingly, in one aspect the invention provides a pharmaceutical composition for the treatment of mammalian neoplasia comprising a non-lethal anti-neoplasia effective amount of a verotoxin, preferably, verotoxin 1, or the pentameric B subunit of verotoxin and a suitable pharmaceutically acceptable diluent, adjuvant or carrier therefor.

The invention preferably provides a pharmaceutical composition and method of treatment for mammalian skin cancers, brain cancers and ovarian cancer.

15 In a further aspect the invention provides a process for the manufacture of a pharmaceutical composition for the treatment of mammalian neoplasia, said process comprising admixing verotoxin or the pentameric B subunit of verotoxin with a pharmaceutically acceptable carrier, adjuvant or diluent therefor.

20 The present invention provides selective, specific cancer treatments wherein verotoxin or the pentameric B subunit of verotoxin selectively binds with Gb₃ in Gb₃-containing cells. This is in contrast to the use of broad spectrum anti-neoplastic agents such as most chemotherapeutic agents, in that non-Gb₃ containing cells are not affected by verotoxin. The present invention thus provides a most beneficial, cell-selective, therapeutic treatment.

25 The treatment is of value against cutaneous T-cell lymphomas, particularly, Mycosis Fungoides, sezary syndrome and related cutaneous disease lymphomatoid papilosis. For example, Mycosis fungoides lesions in humans have been cleared without any observed adverse systemic effects by the application of VT1 (5ng in 2 ml. solution) by interdermal injection in patients.

30 In a further aspect, the invention provides a method of treating mammalian neoplasia comprising treating said mammal with a non-lethal anti-neoplasia effective amount of

a verotoxin, preferably Verotoxin 1 or the pentameric B subunit of verotoxin.

The verotoxin or its B subunit may be administered to the patient by methods well-known in the art, namely, intravenously, intra-arterially, topically, subcutaneously, by ingestion, intra-muscular injection, inhalation, and the like, as is appropriately suitable to the disease. For treatment of a skin cancer, sub-cutaneous application is preferred.

In the practice of the present invention, Verotoxin 1 has been injected intramuscularly into a patient with advanced ovarian carcinoma. No adverse affects were monitored on lymphocyte or renal function and a serum tumour marker was found to continue to rise when the patient was treated with relatively high doses of Verotoxin 1. This tumour was refractory to all conventional cancer therapies. No effect was found on hemoglobin levels.

The verotoxin or its B subunit is, typically, administered in a suitable vehicle in which the active verotoxin or B subunit ingredient is either dissolved or suspended in a liquid, such as serum to permit the verotoxin to be delivered for example, in one aspect from the bloodstream or in an alternative aspect sub-cutaneously to the neoplastic cells. Alternative, for example, solutions are, typically, alcohol solutions, dimethyl sulfoxide solutions, or aqueous solutions containing, for example, polyethylene glycol containing, for example, polyethylene glycol 400, Cremophor-EL or Cyclodextrin. Such vehicles are well-known in the art, and useful for the purpose of delivering a pharmaceutical to the site of action.

Several multi-drug resistant cell lines were found to be hypersensitive to Verotoxin 1. For example, multidrug resistant ovarian cancer cell lines SKVLB and SKOVL were more sensitive to VT cytotoxicity than corresponding non-multidrug resistant ovarian cancer cell line SKOV3. Such an observation indicates the possible beneficial effect for patients bearing the SKVLB cell line cancer than those with the SKOV3 cell line under VT treatment. Further, our observed binding of VT1 to the lumen of blood vessels which vascularize the tumour mass, in addition to the tumour cells per se, may result in an anti-angiogenic effect to augment the direct anti-neoplastic effect of verotoxin.

A series of human Gb₃ containing astrocytoma cell lines were tested for sensitivity to VT. Although all cells were sensitive, the sensitivity varied over a 5000-fold range despite approximately equivalent Gb₃ levels. We have found that treatment of the least sensitive cell line with sodium butyrate initiated a 5000-fold increase in VT sensitivity

concomitant with an alteration in intracellular VT targeting.

Thus, we have also found that the efficacy of verotoxin and its B subunit may be significantly enhanced by a prior treatment of the neoplastic cells with a sensitizer, such as sodium butyrate.

5

Brief Description of the Drawings

In order that the invention may be better understood preferred embodiments will now be described, by way of example only, with reference to the accompanying drawings wherein:

10

Fig. 1 shows the selective neutralization of ACP cytotoxicity by anti VT1 and or anti VT1 B subunit but not by anti VT2 antibodies as determined by cell density measurement after 48 hours;

Fig. 2 shows the viability of selected ovarian and breast tumour cell lines to verotoxin concentration;

15

Fig. 3 represents VT1 contained within ACP preparation binding to Gb₃ (and Gb₂).

Fig. 4 represents VT thin layer chromatography overlay of ovarian tumour and ovary glycolipids;

20

Fig. 5 represents VT thin layer chromatography overlay of selected cell line glycolipids;

Fig. 6 represents in three graphs ovarian cell line sensitivity to VT1, VT2 and VT2c;

Fig. 7 represents glioblastoma multiforme cell line sensitivity to VT1, VT2 and VT2c;

25

Fig. 8 represents the distribution of labelled VT1 B subunit (VTB-¹³¹I) administered IP (inter-peridually) in a Gb₃ tumour bearing nude mouse;

Fig. 9 represents the results of a three-day treatment of several human astrocytoma cell lines with VT1;

30

Figs. 10A - 10G represents a graph of the anti-proliferative effects of VT1 on human astrocytoma cells;

Figs. 11A and 11B provide a comparison of SF-539 and XF-498 sensitivity to VT1 holotoxin;

Figs. 12A and 12B represent the detection of the VT-Receptor glycolipid, Gb₃ in human astrocytoma cell lines;

Fig. 13 shows the sensitivity of two astrocytoma cell lines to VT1 after sensitizing culture; and

Fig. 14 shows the sensitivity to the B subunit of verotoxin VT1 of the two cell lines used in tests shown in Fig. 13.

Detailed Description of the Invention

Experimental

The isolation and purification of verotoxins VT1, VT2 and VT2c have been earlier described.

Verotoxin 1 was prepared genetically from the high expression recombinant E. coli pJB28, J. Bacteriol 166:375 and 169:4313. The generally protein purification procedure described in FEMS Microbiol. Lett. 41:63, was followed.

Verotoxin 2 was obtained from R82, Infect. Immun. 56:1926-1933; (1988); and purified according to FEMS Microbiol. Lett. 48:379-383 (1987).

Verotoxin 2c was obtained from a clinical strain E32511 and purified according to FEMS Microbiol. Lett. 51:211-216 (1988).

VT1 B subunit was prepared according to Ramotar (24). VTs were aliquoted in PBS and stored at 70°C. The appropriate dilution for the treatment of astrocytoma cell lines was prepared freshly in media and added to the cells.

Purification of VT1 from JB28

Pellet Preparation may be conducted as follows:

1. Prepare 6 x 1L LB broth in 3 x 5L jugs (media) and autoclave.
Add carbenicillin to give a 100 µg/ml final conc. when cool.

2. Seed at least 6 ml of penassay (tubes in cold room) + 100 µg/ml carbenicillin with JB28 and incubate O/N @ 37°C.
3. Seed jugs (1 ml seed/litre broth) next morning and incubate for 24 hours at 37°C at 200 rpm (vigorous shaking).
- 5 4. Spin down bugs at 9K for 15 min. at 4°C and scrape pellet into a freezer bag for future use. Freeze at -70°C.

Preparation of Crude Toxin Extract:

- 10 1. Retrieve pellet and dump into beaker. Resuspend in 400 ml of PBS containing 0.1 mg/ml polymyxin B, 50 mg PMSF using a blender. Blend thoroughly then sonicate on ice for - 1 minute to disperse further.
2. Incubate in shaking incubator, 200 rpm, or with vigorous stirring @37°C for 1 hour.
3. Spin down cells @ 9K for 15 minutes.
- 15 4. Pour off supernatant and keep. Resuspend pellet in 400 ml PBS with 0.1 mg/ml polymyxin B and PMSF. Blend and sonicate as before.
5. Incubate with vigorous shaking/stirring at 37°C for 1 hour.
6. Spin at 10K for 15 minutes and save supernatant.
7. The supernatants should be quite yellow and the bacterial pellet should become more fine and diffuse with each extraction step.
- 20 8. Filter the combined supernatants through Whatman* filter paper than through a glass fibre filter to clarify. This step is optional, but will greatly speed the concentration step.
9. Amicon* the combined supernatants at 70 psi (max.) using a YM10 membrane (takes about 200 hours) to concentrate to < 50 ml.

25

Chromatography:

Hydroxylapatite

- 30 1. Equilibrate hydroxylapatite column with 10mM K or Na phosphate (several column volumes).

* Trade-mark

2. Load sample and wash with equilibration buffer until absorbance of effluent is negligible.
3. Add 2 column volumes (150 ml) of 100mM K phosphate (until yellow-coloured fractions emerge) and collect 3 ml fractions.
4. Wash column with 500mM K phosphate and re-equilibrate with 10mM K phosphate. Add 0.05% sodium azide.

Chromatofocussing

5. Measure fractions (A_{280}) and Pool peak fractions from HA.
6. Dialyse against 2L 0.025M imidazole-HCl pH 7.4 O/N. Also equilibrate the chromatofocussing column O/N with the same (300 ml).
7. Load sample and follow with 400 ml polybuffer-HCl pH 5.0 (50ml polybuffer 74 + 350ml dH₂O, a 1:7 dilution, -pH to 5.0 with HCl). NOTE: make sure the sample is equilibrated to the temperature that the column will be run at (usually room temperature) prior to loading. If the column is to be run at 4° then buffers must be pH'd at 4°C and the column equilibrated at this temperature.
8. Collect 1 ml fractions and test them for A_{280} and pH.
9. Plot the A_{280} and pool peak fractions at about pH 6.8 for VT-1 (pool side peaks separately).
10. Clean column with 100 ml 1M NaCl. if really dirty follow with 100 ml 1M HCl, but quickly equilibrate column with imidazole. Store column in 20% ethanol in 25mM imidazole.

Cibachron blue

11. Equilibrate cibachron blue with 10mM Na phosphate buffer, pH 7.2 (100ml).
12. Load sample directly from CF and follow with 60ml of same buffer.
13. Elute with 0.5M NaCl in above buffer and collect fractions.
14. Test fractions for A_{280} and cytotoxicity and pool appropriate ones.
15. Clean column with 25ml each of 8M Urea in wash buffer and 1M NaCl in wash buffer.
16. Reequilibrate column with 10mM phosphate containing 0.1% azide.

17. Dialyse peak fractions against wash buffer with one change.
18. Lyophilize and resuspend in 1ml dH₂O.
19. Do protein assay and run SDS-PAGE to check purity.

5 Solutions:

HA column

potassium phosphate buffer (0.5M stock)

17.42g K₂HPO₄ up to 300 ml with dH₂O

6.8g KH₂PO₄ pH 7.2 with KOH

10 CF column

imidazole buffer

0.851g/500 ml H₂O

pH 7.4 with HCl

15 CB column

sodium phosphate buffer (Wash buffer-WB)

0.71g/500ml Na₂HPO₄

pH 7.2 with HAc

degas

20

Elution buffer

Cleaning Buffers

2.922g NaCl/100 ml WB

12.012g Urea/25 ml WB

1.461g NaCl/25ml WB

Purification of VT2 from R82

25

Pellet Preparation:

1. Prepare 3 x 2L penassay broth (Antibiotic Meida 3, DIFCO, pH 7.0) in 3 x 5L jugs and autoclave at 121°C for 20 minutes. Allow broth to cool to room temperature before use.
2. Seed minimum 3 x 2ml of penassay broth containing 75 µg/ml carbenicillin (Disodium salt, SIGMA) with R82 and incubate overnight at 37°C, with shaking.

30

* Trade-mark

3. Add 50 µg/ml carbenicillin to each of the 5L jugs (from step 1). Seed each jug with 2 ml of seed (step 2) and incubate for 24 hours at 37°C with shaking of approximately 120 rpm.
4. Heat incubator to 45°C and incubate for 30 minutes.
5. Reduce temperature to 37°C and incubate for another 3 hrs.
6. Spin down culture solution at 9,000xg for 15-20 min at 4°C. Discard supernatant and store pellets at -20°C.

Preparation of Crude Toxin Extract:

1. Resuspend pellets in 100 ml of PBS (phosphate buffered saline, OXOID; pH 7.3).
2. Add 0.3 mg/ml PMSF (phenylmethyl-sulfonyl fluoride, SIGMA) dissolved in 0.5 ml acetone to pellet solution. Let acetone evaporate. Sonicate on ice at highest output possible for 5 min or until an homogeneous solution is obtained.
3. Spin down cell at 9,000xg at 4°C for 20 min. Discard pellets.
4. Concentrate supernatants using ultrafiltration (Model 8400 standard infiltration cell, AMICON) with N₂ no higher than 70 psi and using a 10,000 MW cutoff membrane filter (YM10 membrane, AMICON).
5. Using 12-14,000 MW cutoff tubing (SPECTRAPOR) (now and in all dialysis steps), dialyse toxin solution against 4L of 10mM potassium phosphate overnight, with stirring at 4°C.

Chromatography:

Hydroxylapatite (HA)

1. Equilibrate hydroxylapatite column (BSA binding capacity: 32 mg/g, approximately 113 ml volume; CALBIOCHEM * (BEHRING DIAGNOSTICS)) with 2 column volumes of 10mM potassium phosphate.
2. Load sample and follow with 1 column volume 10mM potassium phosphate.
3. Add 2 column volumes of 200mM potassium phosphate and collect 2 ml fractions. The fractions containing the toxin should be coloured differently from the other fractions.

* Trade-mark

4. Wash column with 1 column volume of 500mM potassium phosphate and reequilibrate with 1 column volume of 10mM potassium phosphate. Add azide to the top of the column for storage.

5 Chromatofocussing (CF)

5. Pool peak fractions from HA column either by colour or by cytotoxicity test on Vero cells (10-fold dilutions).
6. Dialyse pooled fractions against 4L 0.025M Histidine-HCl pH 6.2 (SIGMA) overnight. Also equilibrate the chromatofocussing column (PBE (polybuffer exchanger) 94, 1.5 cm diameter, 57 ml volume; PHARMACIA) overnight with the same buffer (300 ml).
- 10 7. Load sample and follow with 400 ml polybuffer-HCl pH 4.0 (50 ml polybuffer 74 (PHARMACIA) + 350 ml dH₂O - pH to 4.0 with HCl).
8. Collect 2 ml fractions and test the pH of each fraction. Once the pH has dropped to 3.95, stop collecting fractions. Test the fractions using absorbance of 280 nm or by cytotoxicity on Vero cells (10-fold dilutions).
- 15 9. Pool peak fractions, and return pH to 7.0 using 1N NaOH.
10. Clean column with 200 ml 1M NaCl. If dirty follow with 100ml 1M HCl, but quickly equilibrate column with 0.025M imidazole, otherwise equilibrate with 24% EtOH-H₂O.

20 Cibachron blue (CB)

11. Equilibrate cibachron blue (2 cm diameter, 82 ml volume, PIERCE) with 100 ml of 10mM sodium phosphate buffer (wash buffer).
12. Load sample and follow with 60 ml of wash buffer.
13. Elute with 0.5M NaCl in wash buffer and collect 2 ml fractions.
- 25 14. Test fractions for absorbance at 280 nm using the elution buffer as a blank and cytotoxicity on Vero cells and pool appropriate fractions.
15. Clean column with 25 ml each of 8M Urea in wash buffer and 1M NaCl in wash buffer.
16. Reequilibrate column with 100 ml of wash buffer and add azide to the top of the column for storage.
- 30 17. Dialyse peak fractions against 4L 0.01M Tris-CL (pH 7.0, SIGMA).

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18. Lyophilize sample and resuspend in 1-2 ml dH₂O (OPTIONAL).
19. Do protein assay (BCA Protein assay reagent, PIERCE) and run SDS-PAGE gel (Schagger, H. and von Jagow, G.; Analytical Biochem 166, 368-379 (1987): 10% T table 2; first line table 3) to check purity.

5

Solutions:

HA Column

potassium phosphate buffer (0.5M stock)

10 17.42g K₂HPO₄ up to 300 ml with dH₂O
6.8g KH₂PO₄ pH 7.2 with KOH

CF column

Histidine buffer (0.025M)

2.0g/500 ml H₂O
15 pH 6.2 with HCl

CB column

Sodium phosphate buffer (Wash buffer-WB)

0.71g/500ml Na₂HPO₄
pH 7.2 with HAc
20 degas

Elution buffer (0.5M)

Cleaning Buffers

2,922g NaCl/100ml WB 12.01g Urea/25 ml WB
1.46 NaCl/25 ml WB
25 0.01 M Tris
4.84 g Trizma Base
4 L ddH₂O
pH to 7.2 with HCl

30 Purification of VT2c from E32511

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Pellet Preparation:

1. Prepare 3 x 2L penassay broth (Antibiotic Media 3, DIFCO; pH 7.0) in 3 x 5L jugs and autoclave at 121°C for 20 minutes. Allow broth to cool to room temperature before use.
2. Seed minimum 3 x 2 ml of penassay broth with E32511 and incubate overnight at 37°C.
3. Add 0.2 µg/ml Mitomycin C (1 ml of 0.4 mg/ml) (add 5 ml of ddH₂O to the vial) to each of the 5L jugs (from step 1). Seed each jug with 2 ml of seed (step 2) and incubate for 6 hrs at 37°C with shaking of approximately 120 rpm. It is very important to stagger the incubation by about 45 min/flask because the toxin begins to deteriorate after 6 hour exposure to Mitomycin C.
4. Spin down culture solution at 9,000xg for 15-20 min at 4°C. Discard supernatant and store pellets at -20°C.

Preparation of Crude Toxin Extract:

1. Resuspend pellets in 150 ml of PBS (Phosphate buffered saline, OXOID; pH 7.3).
2. Add 0.3 mg/ml PMSF (phenylmethyl-sulfonyl fluoride, SIGMA) dissolved in 0.5 ml acetone to pellet solution. Let acetone evaporate. Sonicate on ice at highest output possible for 3 min or until an homogeneous solution is obtained.
3. Add 0.1 mg/ml polymyxin B sulphate (Aerosporin, BURROUGHS WELLCOME INC.; 500,000 units) to solution and incubate with gentle shaking at 37°C for 1 hr.
4. Spin down cells at 9,000xg at 4°C for 20 min (to remove all cells and cell debris from solution).
5. Decant supernatant and store at 4°C. Resuspend pellet in 75 ml PBS and add 0.1 mg/ml polymyxin B.
6. Incubate with gentle shaking at 37°C for 1 hr.
7. Spin down cell at 9,000xg at 4°C for 20 min and pool supernatants (from step 5). Discard pellets.

The next few steps should preferably be done at 4°C:

8. Add crystalline ammonium sulphate very slowly, with stirring to pooled supernatants to

30% saturation.

9. Let stir for 20 min and then remove precipitate by centrifugation (10000g for 10 min).
10. Add crystalline ammonium sulphate very slowly, with stirring to pooled supernatants to 70% saturation.
11. Let stir for 20 min and then centrifuge at 10000g for 10 min.
12. Resuspend pellet from step 11 in 15 ml of 0.01M Potassium phosphate buffer.
13. Using 12-14,000 MW cutoff tubing (SPECTRAPOR) (now and in all dialysis steps), dialyse toxin solution against 4L of 10mM potassium phosphate overnight, with stirring at 4°C.

Chromatography:

Hydroxylapatite (HA)

1. Equilibrate hydroxylapatite column (BSA binding capacity: 32 mg/g, approximately 113 ml volume; CALBIOCHEM (BEHRING DIAGNOSTICS)) with 2 column volumes of 10mM potassium phosphate.
2. Load sample and follow with 1 column volume 10mM potassium phosphate.
3. Add 2 column volumes of 100mM-200mM potassium phosphate and collect 2 ml fractions. The fractions containing the toxin should be coloured differently from the other fractions.
4. Wash column with 1 column volume of 500mM potassium phosphate and reequilibrate with 1 column volume of 10mM K phosphate. Add azide to the top of the column for storage.

Chromatofocussing (CF)

5. Pool peak fractions from HA column either by colour or by cytotoxicity test on Vero cells (10-fold dilutions).
6. Dialyse pooled fractions against 4L 0.025M imidazole-HCl pH 7.4 (SIGMA) overnight. Also equilibrate the chromatofocussing column (PBE (polybuffer exchanger) 94, 1.5 cm diameter, 57 ml volume; PHARMACIA) overnight with the same buffer (300 ml).

7. Load sample and follow with 200 ml polybuffer-HCl pH 5.0 (25 ml polybuffer 74 (PHARMACIA) + 175 ml dH₂O - pH to 5.0 with HCl).
8. Collect 2 ml fractions and test the pH of each fraction. Once the pH has dropped to 5.95, stop collecting fractions. Test the fractions for cytotoxicity on Vero cells (10-fold dilutions).
9. Pool peak fractions.
10. Clean column with 200 ml 1M NaCl. If really dirty follow with 100 ml 1M HCl, but quickly equilibrate column with 0.025M imidazole.

Cibachron blue (CB)

11. Equilibrate cibachron blue (2 cm diameter, 82 ml volume, PIERCE) with 100 ml of 10mM sodium phosphate buffer (wash buffer).
12. Load sample and follow with 60 ml of wash buffer.
13. Elute with 0.5M NaCl in wash buffer and collect 2 ml fractions.
14. Test fractions for absorbance at 280 nm using the elution buffer as a blank and cytotoxicity on Vero cells and pool appropriate fractions.
15. Clean column with 25 ml each of 8M Urea in wash buffer and 1M NaCl in wash buffer.
16. Reequilibrate column with 100 ml of wash buffer and add azide to the top of the column for storage.
17. Dialyze peak fractions against 4L 0.01M Tris-CL (pH 7.0, SIGMA).
18. Lyophilize sample and resuspend in 1-2 ml dH₂O (OPTIONAL).
19. Do protein assay (BCA Protein assay reagent, PIERCE) and run SDS-PAGE gel (Schagger, H. and von Jagow, G.; Analytical Biochem 166, 368-379 (1987): 10% T table 2; first line table 3) to check purity.

Solutions:

HA column

potassium phosphate buffer (0.5M stock)

17.42g K₂HPO₄

up to 300 ml with dH₂O

6.8g KH₂PO₄

pH 7.2 with KOH

CF column

imidazole buffer (0.025M)

0.851g/500 ml H₂O

pH 7.4 with HCl

5

CB column

sodium phosphate buffer (Wash buffer-WB)

0.71g/500 ml Na₂HPO₄

pH 7.2 with HAc

degas

10

Elution buffer

Cleaning buffers

2.922 g NaCl/100ml WB

12.012g Urea/25ml WB

1.461g NaCl/25ml WB

0.01 M Tris

4.84 g Trizma Base

15

4 L ddH₂O

pH to 7.2 with HCl

Affinity purification of verotoxins

20

500µg globotriaosyl ceramide in 1 ml chloroform was mixed and dried with 1g of dried celite. The chloroform was evaporated and the celite suspended in PBS and poured in a column. Crude polymyxin extract 20 ml (25 mg protein) the toxin producing *E. coli* was applied to the column and incubated at room temp for 15 mins. The column was washed with PBS and purified verotoxin eluted with 10 ml 1M Tris pH 9.6. The eluate was neutralized and dialysed. This method is applicable for purification of all verotoxins. (Boulanger, J., Huesca, M., Arab, S and Lingwood, C.A. "Universal method for the facile production of glycolipid/lipid matrices for the affinity purification of binding ligands" Anal Biochem 217: 1-6 [1994])

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Preparation of verotoxin 1 doses

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VT1 was purified from the *E. coli* strain as previously described which overexpresses the cloned toxin genes. The purified toxin was free of endotoxin contamination.

The protein concentration of this batch of verotoxin was determined and the toxin aliquoted and stored at -70°C.

To prepare VT1 doses for patients, VT1 was diluted into injection grade sterile saline containing 0.2% v/v of the patient's own serum. 210 µl of sterile patient serum was added to 10 ml of sterile injection saline and 93.9 ml of purified VT1 (6.7 g/ml) added to give a final toxin concentration of 62.5 ng/ml or 12.5 ng per 0.2 ml. dose. The final toxin preparation was sterile-filtered using a 0.2 µm syringe filter and dispensed in 2 ml aliquots into 10 ml vials. One working vial may be stored at 4°C and the remaining vials frozen until needed.

FITC labelling of VT1: FITC was added directly to VT1 (in a 1:1, w/w ratio) in 0.5M Na₂CO₃/NaHCO₃ conjugated buffer pH 9.5 and the mixture gently rotated for 1.2 hours at room temperature. Free FITC was removed by centrifugation.

Fluorescent Staining of Sections: Samples of surgically removed ovarian tumours were embedded in OCT compound, flash frozen in liquid nitrogen, and stored at -70°C until use. Five µm sections of frozen sample were thawed, allowed to dry and stained with FITC-labelled VT1 in PBS (0.5 mg/ml) containing 0.1% BSA for 1 h at room temperature. Sections were extensively washed with PBS and mounted with mounting medium containing DABCO*. Sections were observed under a Polyvar fluorescent microscope.

Fluorescent Staining of Cells: Cells growing on coverslips were washed once with PBS, fixed for 2 min at room temperature with 2% formalin rinsed with PBS twice and incubated with FITC-VT1 for 1h at room temperature. The cells were washed 5 times with PBS, mounted with DABCO and observed under a Polyvar fluorescent microscope.

Quantification of VT1 antitumour activity: SKOV3 (drug sensitive human ovarian cell line), SKOVLC (SKOV3, resistant to Vincristine, and SKOVLB (SKOV3, resistant to Vinblastine) were each grown in α - MEM supplemented with 10% fetal calf-serum and tested for their sensitivity to VTs. Equal numbers of cells (approximately 1000 per/ml of media) were added to the wells of Linbro 96 well plate. 10-fold dilution of VTs were tested in triplicate and

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incubated for 48h at 37°C in a humidified atmosphere containing 5% CO₂. Cells were then fixed with 2% Formalin, stained with Crystal Violet, and read with ELISA plate reader.

To quantify the anticancer activity of VT1, SKOV3, SKOVLC, and SKOVLB (human ovarian cell line) were incubated with 10-fold dilution of VT1 for 48h. SKOVLC & SKOVLB (drug resistant cell lines) are more sensitive to VT1 antitumour activity than SKOV3.

Preparation of ¹³¹I-VT1B

This material may be made by the following procedure.

1. Dissolve 20 mg of iodogen in 2.0 ml of chloroform (10 mg/ml). Make a 1:10 dilution by adding 0.25 ml of the 10 mg/ml solution to 2.25 ml chloroform (1 mg/ml).
2. Dispense 20 ul of this dilute solution into a clean, dry sterilized glass tub. Add 500 ul of chloroform and evaporate to dryness under N₂.
3. Add 1.5 mg. in 0.66 ml of VT1B subunit to the test tube.
4. Add 5 MCi of ¹³¹I sodium iodide in 100 ul. Allow labelling to proceed for 10 mins.
5. Wash a PD-10 column with 25 ml of Sodium Chloride Injection USP.
6. Dilute ¹³¹I-VT1B to 2.5 ml total volume with 1% HSA in Sodium Chloride Injection USP. Load onto PD-10 column. Elute column with 3.5 ml 1% HSA in saline.
7. Measure ¹³¹I activity of eluant and column to determine LE. Draw up pooled fractions into a syringe with spinal needle attached. Detach spinal needle and attach Millex ^{*}GV filter.
8. Filter into a sterile 10 ml multidose vial. Note volume filtered and assay vial for ¹³¹I in dose calibrator. Calculate concentration.
9. Draw up 0.1 ml of ¹³¹I-VT1B and dispense 0.05 ml into each of two 5 ml sterile multidose vials (one for sterility test and one for pyrogen test). Vials already contain 2 ml saline (=1:50 dilution).
10. Determine RCP by PC (Whatman No. 1) in 85% MeOH and by size exclusion HPLC.
11. Conduct sterility and pyrogen tests.

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Astrocytoma Cell Lines, Endothelial Cells and Culture Conditions:

Six permanent human malignant astrocytoma cell lines (SF-126, SF-188, SF-539, U 87-MG, U 251-MG, and XF-498) were selected for study. SF-126, SF-188, and SF-539 were kindly provided by Dr. Mark Rosenblum, Henry Ford Hospital. U 87-MG and U 251-MG were kindly provided by Dr. Jan Ponten, University of Uppsala, Sweden; and XF-498 was a gift of Dolores Dougherty, University of California San Francisco. Astrocytoma cells were cultured in alpha-MEM, nonessential amino acids, glutamine, gentamycin, and 10% heat-inactivated fetal bovine serum. The cultures were incubated at 37°C and equilibrated in 5% CO₂ and air. Cells were harvested with 0.25% trypsin (Gibco, Santa Clara, CA) in Ca⁺⁺ and Mg⁺⁺ free Hank's balanced salt solution and were subcultured weekly.

Human capillary endothelial cells were isolated after the method of Costello (25) and were derived from samples of normal human brain taken from patients undergoing neurosurgical procedures for epilepsy, trauma, and resection of arteriovenous malformations. The capillary cells were grown as described above in media supplemented with 15 µg/ml endothelial growth factor (Sigma, St. Louis) (26). The endothelial origin of the cells in culture was established by immunocytochemical analysis using anti-human factor-VIII-related antigen antisera (Dako, Santa Barbara, CA) as described previously (27).

Approximately 1.5×10^4 cells were added to 24-well plates and incubated in alpha-MEM in 5% CO₂ at 37°C. After 24 hours, the growth medium was replaced with medium containing various concentrations of the holotoxin VT1 (0, 0.1, 5, 50, and 100 ng/ml). The treated astrocytoma cell lines and endothelial cells were trypsinized and counted at intervals throughout the growth curve. Cell viability was assessed by trypan blue dye exclusion. Cell counts were plotted again time for the various concentrations of VT1 and B subunit. For each time point analyzed, the wells were set-up in triplicate.

For selected cell lines, the B subunit of VT1, VT2, and VT2c was added alone to the astrocytoma cells at same concentrations listed above. In these experiments, a single dose of VT1, VT2, and VT2c was added to confluent astrocytoma cells in microtiter wells. Cell survival at 72 hours was monitored by staining with 0.1% crystal violet, and measuring the optical density at 590 nm using a Dynatek microtiter plate reader.

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VT Receptor Analysis of Human Astrocytoma Cells:

Cultured human astrocytoma cells were homogenized in a minimum volume of PBS and extracted with 20 volumes of a 2:1 by volume chloroform:methanol solution. The extract was partitioned against water and the lower phase partitioned again against theoretical upper phase. The lower phase was dried completely and dissolved in a known volume of 2:1 chloroform:methanol. The presence of Gb₃ was detected by TLC overlay binding with VT1. Astrocytoma lower phase and standard Gb₃ from human kidney each were separated by TLC [(chloroform:methanol:water = 65:25:4 (v/v/v))]. The TLC plates were dried and blocked with 1% gelatin in water at 37°C overnight. Then they were washed three times with 50 mM TBS (Tris Buffer Saline) for 5 min and incubated with 0.1 µg/ml VT1 for 1 hour. After further washing with TBS, the plates were incubated with a mouse monoclonal PH1 and anti-VT1 antibody (2 µg/ml), followed, after washing, by peroxidase-conjugated goat anti-mouse antibody or peroxidase conjugated goat anti-rabbit antibody as appropriate. Finally, the plates were washed with TBS, and VT1 binding was visualized with 4-chloro-1 naphthol peroxidase substrate. A similar plate was prepared and stained with orcinol carbohydrate spray for comparison.

Nuclear staining with propidium iodide:

SF-539 cells grown on the cover slips overnight were incubated at 37°C with VT1 B-subunit (50 µg/ml) for 1.5 hrs or 10 hrs and fixed (with 1% paraformaldehyde for 3 minutes), permeabilized with 0.1% Triton X in 100 mM PBS for 5 min, and stained with 5 µg/ml propidium iodide (sigma). After extensive wash with 50 mM PBS, the fixed cells were mounted with DABCO (1,4-Diazabicyclo-Octane, sigma), and nuclear staining observed under incident uv illumination.

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Flow Cytometry:

Apoptosis of astrocytoma cells, incubated with 10 ng/ml of VT1 for 24-36 hrs in the presence of 10% bovine fetal serum was analyzed on an Epics^{*} Profile Analyzer (Coulter Electronics, Pathology, University of Toronto). After treatment, cells were trypsinized and the 200Xg centrifuged cell pellet was suspended in 1ml of hypotonic fluorochrome solution of 50 µg/ml propidium iodide (sigma) and stained for 30 min at 4 C. To remove RNA prior to staining, cells were treated with 100 µl of 200 µg/ml DNase-free RNase A at 37 C for 30 min. Cell cycle distribution was determined using manual gating. Flow cytometric quantitation of apoptotic cells within the propidium iodide-stained population was performed. Debris and dead cells were excluded on the basis of their forward and side light-scattering properties. Astrocytoma cells grown simultaneously in the absence of VT1 served as controls.

Ultrastructural Analysis of VT-treated Astrocytoma Cells:

Cells were cultivated on a transferable 9 mm cyclopore membrane (0.45 µm pore size, Falcon) to form a confluent monolayer and were incubated at 37°C with VT1 (10 ng/ml). Cells were fixed at room temperature by addition of 1.6% glutaraldehyde to the well and then incubated in 0.066 M Sorensen buffer (pH 7.4) containing 1.5% glutaraldehyde for 1 h at 4°C. After 2 h of washing with 0.1 M phosphate buffer, cells were post-fixed in 2% osmium tetroxide in the same buffer. After dehydration in graded ethanols and propylene oxide, Epon embedding and uranyl-lead staining were performed. Thin sections were examined in a Philips EM 400 electron microscope and ultrastructural features of apoptosis was analysed.

Fig. 1 relates to the neutralization of ACP cytotoxicity by anti-VT. KHT cell monolayers were incubated with 35 ng/ml ACP from E.coli HSC₁₀, or 10pg/ml VT1, VT2 or VT2c in the presence of monoclonal anti-VT1(PH1), monoclonal anti VT2 or polyclonal rabbit antiVT1 B subunit. The cells were incubated for 72 hours at 37°C and viable adherent cells were detected by fixation and staining with crystal violet. Cytotoxicity of VT1 and ACP was completely neutralized in the presence of anti VT1 or anti VT1B subunit (anti-VT2 serum had no effect).

From measurement of the cytotoxic assay of ACP on vero cells (cells from

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Africa green monkey kidney that are very sensitive to verotoxin), relative to a pure VT1 standard, it was estimated that the ACP preparation contained 0.05% VT1. This concentration of purified VT1 was as effective as ACP in inhibiting the growth of various tumour cell lines in vitro (Fig. 2). Thus, VT1 mimics the anti-neoplastic effect of ACP in vitro. VT1 was tested for the ability to inhibit the metastases of KHT fibrosarcoma cells in the mouse model as had been previously reported for ACP. The equivalent dose of VT1 was as effective as ACP, reducing the number of lung metastases to background levels, following a primary subcutaneous tumour inoculum (Table 1).

Table 1. Response of KHT cells, growing as lung modules, to treatment with VT-1 or ACP.

GP	TREATMENT	# OF MICE	# OF LUNG NODULES/MOUSE	MEAN	WT LOSS /GAIN*
EXPT 1					
1	Control	9	34,24,39,47,28,32,26,29,34	32.6	+5%
2	ACP-0.25 ug/mouse	4	12,31,25,15	20.8	0
3	ACP-1.0 ug/mouse	6	1,2,2,5,1	2.2	0 ** (1 death)
4	ACP-4 ug/mouse	5	0,0,0,0,0	0	-13%
5	VT-1 0.009 ug/mouse	5	29,41,34,29,21	30.8	+5%
6	VT-1 0.036 ug/mouse	5	7,16,29,16,6	14.8	+5%
7	VT-1 0.144 ug/mouse	5	1,4,2,3,1	2.2	+5%
EXPT 2					
1	Control	4	15,12,8,12	11.75	<5%
2	ACP-2 ug/mouse	5	0,1,0,0,0	0.2	<5%
3	VT-1 0.1 ug/mouse	4	0,0	0	<5%*** (2 deaths)
4	VT-1B-0.2 ug/mouse	5	13,14,9,7,19	12.4	<5%
5	VT-1B-10 ug/mouse	5	8,3,9,11	6.8	<5%

Mice were treated with VT-1 or ACP(l-p) 1 day after cell injection (1000 KHT cells/mouse i-v).

Lung nodules counted @ 20 days after cell injection.

* Mean change in gp wi-max during 10 days (Expt 1) or 4 days (Expt 2) after VT-1 or ACP injection. Max wt loss @ days 7-8.

** Death occurred @ days 2-3 after ACP injection

*** Deaths occurred @ days 7-8

Purified VT1 was found to mimic the anti-metastatic effect of ACP on the growth of this tumour from a primary subcutaneous site. Lung metastasis was completely inhibited. Moreover, prior immunization of mice with the purified B-subunit of verotoxin completely prevented any protective effect of ACP when the animals were subsequently treated with the tumour and ACP (Table 2).

Table 2. Response of KHT lung nodules, growing to immunized mice, to treatment with VT1 or ACP.

GP	IMMUNIZATION*	TREATMENT	# OF MICE	# OF LUNG NODULES/ MOUSE	MEAN	WT LOSS/ GAIN*
1	None	None	6	34,47,53, 62,43,52	48.5	<5%
2	None	VT-1 -0,2 ug/mouse	5			5 deaths (dy 6-8)**
3	None	ACP-2.0 ug/mouse	5	0,1,2,0,0	0.6	-8%
4	VT-1B+FA	None	5	43,40,47, 43,23	39.2	-6%
5	VT-1B+FA	VT-1 -0,2 ug/mouse	6	26,44,49, 21,43,37	36.7	<5%
6	VT-1B+FA	ACP-2.0 ug/mouse	6	50,38,33, 41,48,50	43.3	<5%
7	FA only	None	5	44,60,19, 25,40	37.6	<5%
8	FA only	VT-1 -0,2 ug/mouse	5			5 deaths (dy 6-8)***

GP	IMMUNIZATION*	TREATMENT	# OF MICE	# OF LUNG NODULES/ MOUSE	MEAN	WT LOSS/ GAIN*
9	FA only	ACP -2.0 ug/mouse	5	1,1,2,1,0	1	-6%

Mice were treated with VT-1 or ACP(i-p) 1 day after cell injection (1000 KHT cells/mouse).

Lung nodules counted @ 20 days after cell injection (i-v).

*Immunization was 2 injections of VT-1B (10ug/mouse +/- Freund's Adjuvant (FA) given (i-p) 4 weeks and 2 weeks before cell injection.

** Mean change in gp wt - max during 13 days. Maximum weight loss @ day 7-8.

ACP was tested for glycolipid binding by TLC overlay using monoclonal anti-VT1 or anti-VT2c. Anti-VT1 shows extensive binding of a component within the ACP preparation to globotriaosylceramide and galabiosyl ceramide (Fig. 3). This binding specificity is identical to that reported for purified VT1(8). No binding component reactive with anti-VT2 was detected. In Fig. 3 anti VT antibodies were used to detect binding to the immobilized glycolipids. Arrows indicate position of standard (from the top) galabiosyl ceramide, globotriaosyl ceramide and globotetraosyl ceramide. Panel 1-detection using anti VT1, panel 2-detection using anti VT2c.

VT1 demonstrated *in vitro* activity against a variety of ovarian carcinoma cell lines. A large number of primary human ovarian tumour biopsies were screened for the expression of Gb₃ via TLC overlay using purified VT1. It was found that Gb₃ was barely detectable in normal ovary tissue, whereas in all cases a significant increase in expression of Gb₃ was observed in the ovarian carcinoma. Similarly, elevated levels of Gb₃ were found in acites tumour and in tumours that had metastized to the omentum, (Fig. 4) which defines lane 1, ovarian omentum metastasis; lane 2: tumour biopsy; lane 3, tumour biopsy; lanes 3-6, normal ovary; lane 7, human kidney Gb₃ standard. Surprisingly, we have found that multi-drug resistant variants of ovarian tumour cell lines were considerably more sensitive to VT1 cytotoxicity than the drug sensitive parental cell line (Figs 2, 5 and 6). Similar effects had been observed for ACP. Fig. 2 shows human ovarian tumour cell lines sensitive to ACP tested for VT sensitivity.

Human ovarian and breast tumour derived cell lines were tested for VT1 sensitivity wherein ovarian 1, 2, 3, 4 and 5 are denoted □, +, x, ■ and o respectively, and breast-SKBR3, 468♦, 453⊙, 231▲. The cell lines 1-ovarian, 453 and SKBR3, previously shown to be resistant to ACP, were also resistant to up to 20 ng/ml VT1.

The 1, 2, 3 and 4 cells were from ovarian cancer patients; the 453 cells were from a breast cancer patient; 231 and SKBR3 are breast adenocarcinoma cell lines, and 5, SKOV3 and SKOVLB are adenocarcinous ovarian cancer cell lines. The lines 1, 453 and SKBR3, resistant to ACP, were co-resistant to VT1. Fig. 5 shows VT sensitive and resistant cell lines tested for the presence of Gb₃ by VT binding in tlc overlay. Glycolipid from an equal number of cells were extracted and separated by tlc prior to toxin binding. In Fig. 5, lane 1:SKBR3, lane 2:468, lane 3:231, lane 4:453, lane 5 Gb₃ standard, lane 6:SKOV3, lane 7:SKOVLB. Cell lines SKBR3, 468, 231 and 453 are derived from breast tumours. Only 231 is sensitive to VT1. SKOVLB is a multiple drug resistant ovarian tumour cell line derived from SKOV3.

Ovarian tumour cells were highly sensitive to VT (Fig. 3) and contained elevated levels of the VT receptor, Gb₃ (Fig. 4). Breast cancer cells were for the most part, toxin resistant (Fig. 3) and receptor negative (Fig. 5). Low levels of Gb₃ were detected in normal ovarian tissue but these were markedly elevated for the ovarian tumour tissue samples.

The specific elevation of Gb₃ in ovarian tumours as opposed to normal ovary tissue provides the feasibility of using the toxin in the management of this malignancy. Ovarian tumours are often refractory to chemotherapy and prognosis is poor. Indeed, preliminary phase 1 clinical trials using a ACP injected directly into skin malignancies (Mycosis fungoides) have proven successful without adverse systemic effects.

With reference now to Fig. 6, human derived ovarian tumour cell lines were tested for VT1, VT2, and VT2c sensitivity. The cells were grown to confluence in 48-well plates, then incubated for 48 hrs. in the presence of increasing doses of VTs. SKOVLB, the multiple drug resistant variant of SKOV3 ovarian line, showed the most sensitivity to VT's with SKOVLB being the next most sensitive to the VT's.

We have found that both drug resistant cells are approximately 500 to 1000 times more sensitive to verotoxin cytotoxicity than the parental SKOV3 cell line.

Fig. 7 shows the effect after 48 hrs. of treatment of the brain tumour SF-539 cell line derived from a recurrent, right temporoparietal glioblastoma multiform with VT1, VT2, and VT2c. This cell line, as others, was highly sensitive to VT's.

Fig. 8 provides the results from imaging a nude mouse with ^{131}I -VT1B (CPM distribution in different organs). VT1B- ^{131}I cpm distribution in nude mouse with implanted ovarian tumour showed that a considerable amount of radiolabelled VT1B had been concentrated in the ovarian tumour. Only a trace amount of VT1B was located in the brain where the potential VT1 side effect was considered. Since the lung in human adult is not the site of concern for VT1 toxicity this does not present a problem for treatment of human adult with ovarian tumour. In addition the CPM in kidney includes the excreted radiolabelled VT1 B subunit. Accordingly, based on this test, imaging with labelled VT1 B subunit can be a very useful method for screening the susceptible patient to VT1 cytotoxicity.

Fig. 9 shows the sensitivity of a variety of human astrocytoma cell lines to VT1. All these cells contain Gb₃ but show variable sensitivity to VT1 induced cytotoxicity. This suggests that certain astrocytomas will be more susceptible to verotoxin than other astrocytomas. This is important since astrocytomas are very refractory to treatment at the present time and cell sensitivity in vitro to concentrations as low as 5ng per/ml is rare.

Figs. 10A - 10G show the anti-proliferative effects of VT1 on human astrocytoma cells. All astrocytoma cell lines showed at least some inhibition of growth following VT1 treatment. The most sensitive cell line was SF-539 (Fig. 10A), and the least sensitive was SF-126 (Fig. 10F). Human cerebral capillary endothelial cells were largely resistant to the growth-inhibitory effects of VT1 except at high doses (100 ng/ml) (Figs. 10G). U-251 MG and U-87 MG were sensitive to VT1 (Figs. 10B and 10C), whereas XF 498 and SF-188 were somewhat less sensitive to VT1 (Figs. 10D, 10E and 1E) than were U-251 MG and U-87 MG.

Figs. 11A and 11B provide a comparison of SF-539 and XF-498 sensitivity to VT1 holotoxin (upper panel) and B-subunit (lower panel). Forty-eight hrs following the treatment of SF-539 and XF-498 cells in monolayer culture, the percent cell survival was calculated. VT1 was cytotoxic to SF-539 astrocytoma cells at doses as low as 0.01 ng/ml (upper panel). XF-498 cells were resistant to VT1 holotoxin. When the VT1 B-subunit was

employed, only SF-539 was sensitive to this toxin (lower panel).

Figs. 12A and 12B represent the detection of the VT-Receptor glycolipid, Gb₃ in human astrocytoma cell lines. Astrocytoma neutral glycolipids were prepared from 1×10^6 cells and separated by TLC. (A) Glycolipids were visualized by orcinol and bands representing Gb₃ are seen in all astrocytoma cell lines. (B) The same blot was assayed by VT1 overlay. In this study, VT1 binds to Gb₃ extracted from astrocytoma cells as shown (arrow). SF-539 astrocytoma cells showed maximal binding of Gb₃/VT1. Lane 1, U87 MG; lane 2, U251 MG; lane 3, SF-126; lane 4, SF-188; lane 5, XF-498; lane 6, SF-539, lane 7, standard Gb₃ (0.3 ug/ml).

Fig. 13 compares the sensitivity of two astrocytoma cell lines SF539 (sensitive), XF498 (less sensitive) and XF 498, following three days of culture of XF498 in sodium butyrate. It is seen that the sensitivity of XF498 is increased to that or even more than that of the most sensitive cell line SF539. Fig. 14 shows the same effect for the B subunit of verotoxin 1.

Anti-Proliferative Effects of Verotoxin on Human Astrocytoma Cells:

Figs. 10A - 10G show that all astrocytoma cell lines studied were sensitive to VT1. The most sensitive cell line in terms of growth inhibition was SF-539 (Fig. 10A) and the least sensitive was SF-188 (Fig. 10E). When treated with other members of the VT family including VT2, and VT2c, SF539 was growth inhibited. VT-1 was the most potent species (Fig. 11). Interestingly, human cerebral endothelial cells were largely resistant to the growth inhibitory and cytotoxic effects of VT-1 (Fig. 10G). Only when doses as high as 100 ng/ml were used were endothelial cells inhibited.

A comparison between the sensitivity of SF 539 and XF498 for VT1 and VT1 B subunit is shown in Fig. 11A and Fig. 11B. XF498 cells were considerably less sensitive to the B subunit than to the VT-1 holotoxin. By comparison, SF 539 astrocytoma cells were significantly more sensitive to the B subunit alone than were XF 498 astrocytoma cells, since 50% cell death was observed in the presence of 50 ng/ml.

VT-Receptor Analysis of Human Astrocytoma Cells:

The glycolipid profile of the 6 human astrocytoma cell lines analyzed for Gb₃ content as detected with orcinol is shown in Fig. 12A. All of the astrocytoma cell lines expressed significant levels of Gb₃ and showed binding with VT1 in the overlay assay used (Fig. 12B). SF-539 cells expressed the highest levels of Gb₃ with maximal binding to VT1.

Flow Cytometric Analysis:

To determine the extent of astrocytoma cells death by apoptosis, cells were analysed by flow cytometry. SF-539 and XF-498 astrocytoma cells exposed to VT1 (10 ng/ml) revealed the characteristic features of apoptosis. As a result of chromatin condensation and DNA cleavage, apoptotic cells show less propidium iodide fluorescence than viable cells and can be quantified as the "subdiploid" population or pre-G1 position in cell cycle (Fig. 13, arrow head). Presence of cells with fractional DNA content, typical of apoptosis was more marked in SF-539 than XF-498 cells. A cell cycle analysis of the non-apoptotic cell population revealed marked differences in the proportion of cells in the respective phases of the cell cycle. In VT1-sensitive SF-539 cells, a pronounced loss of S phase cells from 33 to 15 and 10% was seen, whereas with the less VT1 sensitive XF-498 cells, the loss of S phase cells observed was only 75 to 69 and 65%. Changes in the proportion of cells in G2-M phase were also seen (Fig. 13).

Propidium iodide stains:

For detection of apoptotic morphology in cells treated with VT1 or VT1 B-subunit, permeabilized SF-539 and XF-498 cells were stained with the DNA-intercalating agent propidium iodide and were analyzed by fluorescence microscopy. VT1 treated cells displayed characteristic features of apoptosis, such as marked reduction in diameter, condensed chromatin. Nuclear segmentation and subnuclear bodies were prominent in cells treated with VT1 B-subunit for 1.5 or 10 hours.

Ultrastructural Analysis of VT-Treated Astrocytoma Cells:

By electron microscopy, VT1-treated astrocytoma cells (SF-539, XF-498) demonstrated characteristic features of apoptosis such as, blebbing of the cytoplasmic membrane, fragmentation of heterochromatin, condensation of the nucleolar membrane, loss of cell junctions and microvilli, nuclear disintegration, and apoptotic bodies.

The results herein show that VT1 inhibits the growth of a series of human astrocytoma cell lines. All cell lines showed significant sensitivity to VT1, contained the Gb₃ receptor for VT, and demonstrated ultrastructural features indicative of apoptosis following VT treatment. These results show that VTs provide the basis of new agents active against human astrocytoma cells. The results show that the most toxin sensitive astrocytoma cell line, SF-539, is also highly sensitive to B subunit induced apoptosis.

Definitive morphological evidence of apoptosis (nuclear shrinkage and chromatin condensation) were observed within 1.5 hrs of toxin or B subunit administration to astrocytoma cells. This is considerably more rapid than has previously been described for induced apoptosis by anticancer drugs. Accumulation of VT1-treated astrocytoma cells in pre-G1 position in cell cycle (Fig. 13) is strong evidence for apoptosis. Additional evidence in support of VT1 causing apoptosis in sensitive astrocytoma cells include nuclear staining with propidium iodide and ultrastructural alterations indicative of apoptosis.

We have found that there was relative insensitivity of human cerebral capillary endothelial cells to VT.

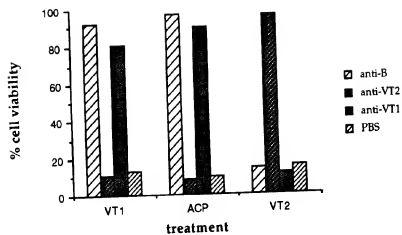
While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope of the invention.

Claims:

1. A pharmaceutical composition for treatment of mammalian neoplasia comprising globotrisylceramide (Gb₃)-containing cells, said composition comprising a non-lethal, antineoplasia effective amount of a verotoxin and a suitable pharmaceutically-acceptable diluent, carrier, or adjuvant therefor, wherein said neoplasia is a brain, breast, or skin cancer.
2. A composition as defined in Claim 1 wherein said verotoxin is verotoxin 1.
3. A composition as defined in Claim 1 wherein said verotoxin is verotoxin 2.
4. A composition as defined in Claim 1 wherein said verotoxin is verotoxin 2c.
5. A composition as defined in Claim 1 wherein said brain cancer is an astrocytoma.
6. A pharmaceutical composition for the treatment of mammalian neoplasia selected from brain, breast or skin cancer, said composition comprising globotrisylceramide (Gb₃)-containing cells said composition comprising a non-lethal, anti-neoplasia effective amount of the pentameric B sub-unit of verotoxin and a suitable pharmaceutically-acceptable diluent, carrier or adjuvant therefore.
7. Use of a pharmaceutical composition for the treatment of mammalian neoplasia comprising globotrisylceramide (Gb₃)-containing cells, said composition comprising a non-lethal, antineoplasia effective amount of a verotoxin and a suitable pharmaceutically acceptable diluent, carrier, or adjuvant therefore, wherein said neoplasia is brain, breast, or skin cancer.
8. A use as claimed in Claim 7 wherein said verotoxin is verotoxin 1.
9. A use as claimed in Claim 7 wherein said verotoxin is verotoxin 2.
10. A use as claimed in Claim 7 wherein said verotoxin is verotoxin 2c.
11. Use of a pharmaceutical composition for the treatment of mammalian neoplasia selected from brain, breast or skin cancer, said composition comprising globotrisylceramide (Gb₃)-containing cells, said composition comprising a non-lethal, anti-neoplasia effective amount of the pentameric B sub-unit of verotoxin and a suitable pharmaceutically acceptable diluent, carrier, or adjuvant, therefore.
12. A use as claimed in Claim 11 wherein said neoplasia is selected from brain, ovarian, breast and skin cancer.
13. A use as claimed in Claim 12 wherein said brain cancer is an astrocytoma.

14. A use as claimed in Claim 7 or claim 11 further comprising treating said mammal with a non-lethal, effective, sensitizing amount of a sensitizer.
15. A use as claimed in Claim 14 wherein said sensitizer is sodium butyrate.
16. A use as claimed in claim 14 wherein said composition is for application of
5 said sensitizer and said verotoxin to the locus of the neoplastic cells.
17. A use as claimed in claim 14 wherein said composition is for application of said sensitizer to the locus of the neoplastic cells.
18. A pharmaceutical composition for the treatment of ovarian cancer comprising globotrisylceramide (Gb₃)-containing cells, said composition comprising a non-lethal,
10 anti-ovarian cancer effective amount of a verotoxin selected from verotoxin 2 and verotoxin 2c and the pentameric B sub-unit of verotoxin and a suitable pharmaceutically-acceptable diluent, carrier or adjuvant, therefor.
19. Use of a pharmaceutical composition for the treatment of ovarian cancer comprising globotrisylceramide (Gb₃)-containing cells, said composition comprising a
15 non-lethal, anti-ovarian cancer effective amount of a verotoxin selected from verotoxin 2 and verotoxin 2c and the pentameric B sub-unit of verotoxin and a suitable pharmaceutically-acceptable diluent, carrier or adjuvant, therefor.

fig 1



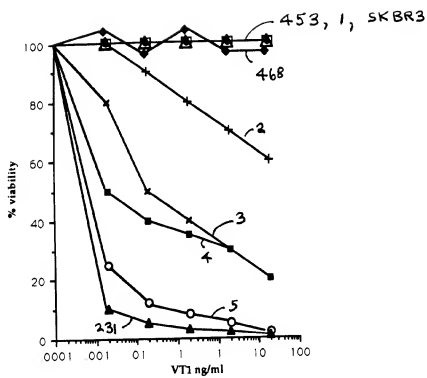


Fig. 2

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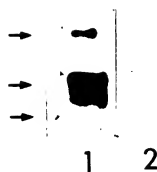


Fig. 3

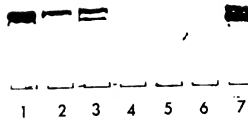
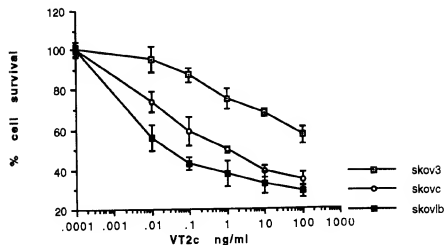
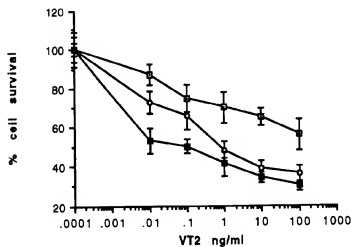
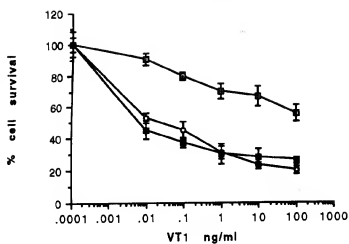


Fig. 4



Fig. 5

Figure 6



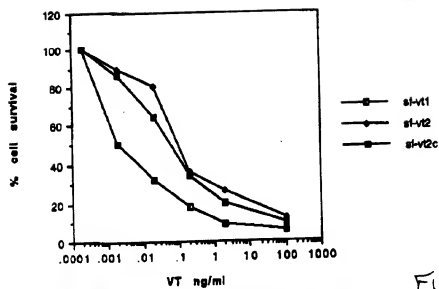


FIG 7

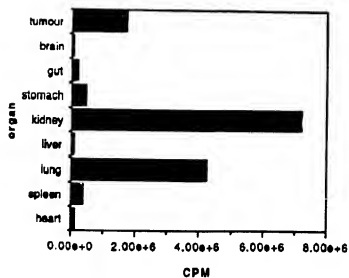


FIG 8

3-day treatment of astrocytoma lines with VT1,

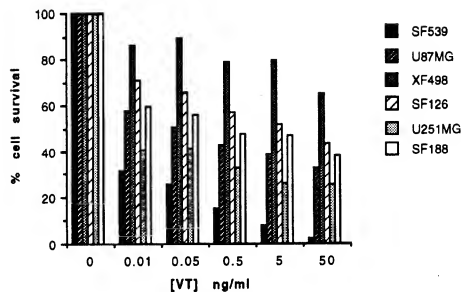


Fig. 9

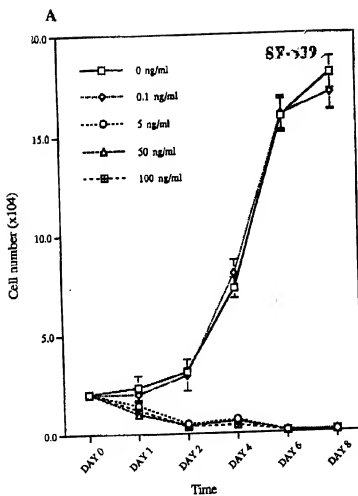


FIG 10A

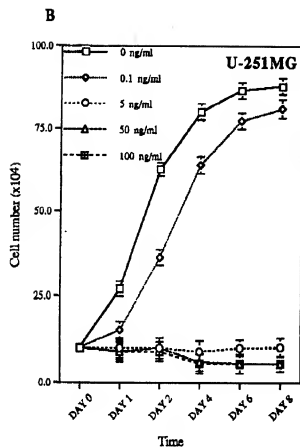


FIG 10 B

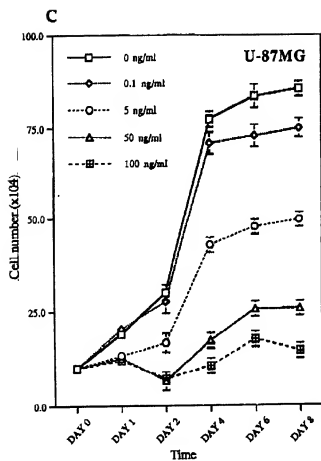


FIG 10 C

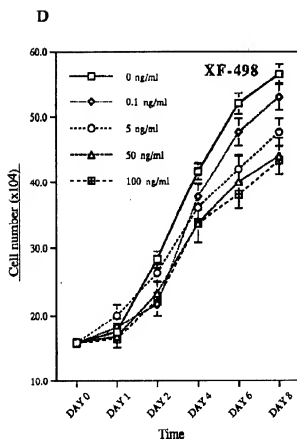


FIG 10

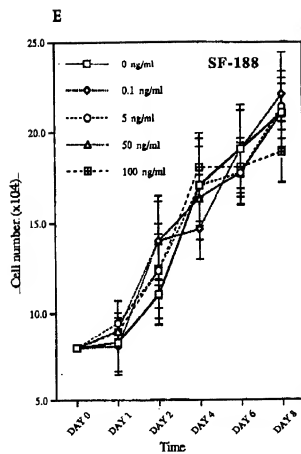


FIG 10E

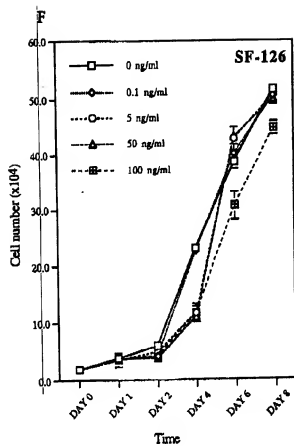


FIG 10 F

G

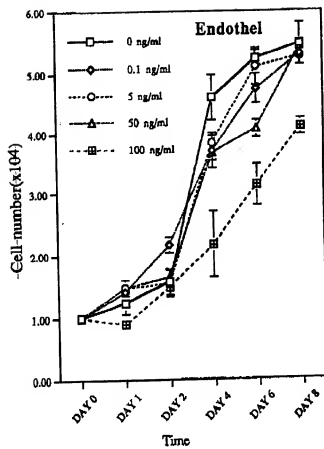


FIG 10G

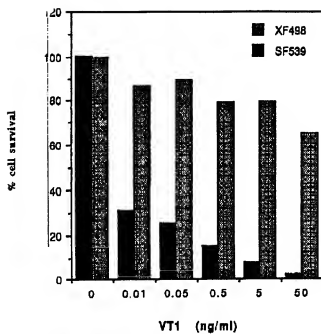


FIG. 11A

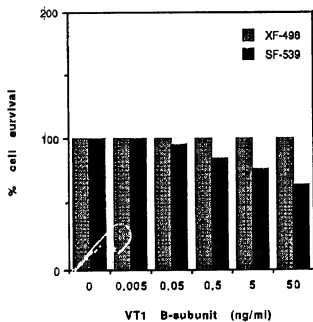


FIG. 11B

FIG. 12

A

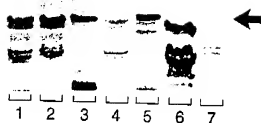
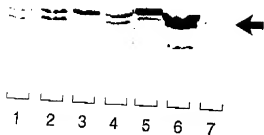


FIG. 12.

B



3-days treatment of cells with VT1

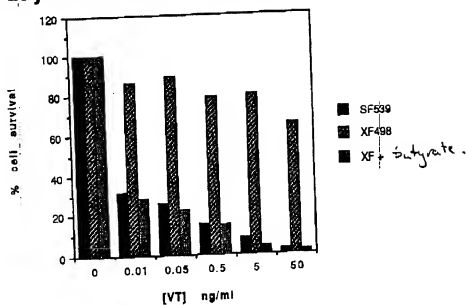


FIG. 13

3-days treatment of cells with VT1 B-subunit

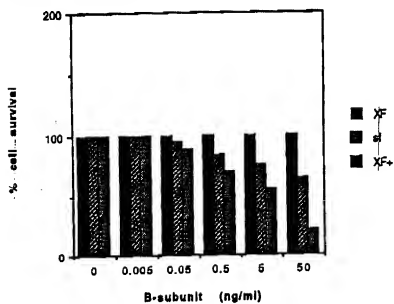


FIG. 14